Allelotyping of Esophageal Squamous-Cell Carcinoma on Chromosome 13 Defines Deletions Related to Family History

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We previously reported that esophageal squamous-cell cancers (ESCC) from Shanxi Province in China show frequent allelic loss on chromosome 13. Moreover, tumors from patients with a positive family history of upper gastrointestinal tumors exhibit more frequent loss of heterozygosity (LOH) on this chromosome than do those from patients without a family history. These results suggest the possibility of a familial ESCC susceptibility gene. To investigate this phenomenon further, we performed an in-depth analysis of allelic-loss data sets from both patients with and without a family history of upper gastrointestinal tumors. Comparisons between deletion frequency and location were made with respect to family history status, risk factors, and clinical/pathologic characteristics of the tumors. The analysis confirmed that tumor LOH was significantly higher in patients with a positive family history than in those who were family-history-negative, and four common deletion regions in these family-history-positive patients were defined. Statistically significant associations were also observed between allelic loss and tumor grade and location, as well as the presence of lymph node metastases. Taken together, these data indicate that a gene or genes on chromosome 13 play an important role in the etiology and progression of ESCC. Published 2005 Wiley-Liss, Inc.†

INTRODUCTION

Esophageal cancer is one of the most common fatal cancers worldwide. There is great geographic variation in the occurrence of this tumor, including exceptionally high-risk areas such as Shanxi Province, a region in north central China with some of the highest esophageal cancer rates in the world (Li et al., 1980; Li, 1982). Previous studies in this high-risk region demonstrated a strong tendency toward familial aggregation (Li and He, 1986; Wu et al., 1989; Hu et al., 1991, 1992), suggesting that genetic susceptibility may play a role in the etiology of esophageal cancer.

Chromosomal regions with frequent allelic loss may point to major susceptibility genes that will assist us in understanding the molecular events involved in esophageal cancer. They may also serve as the basis for the development of markers of genetic susceptibility or screening for early detection of this tumor. Loss of heterozygosity (LOH) on chromosome arm 13q is detected frequently in many types of tumors (Kuroki et al., 1995; Montesano et al., 1996; Eiriksdottir et al., 1998; Hyytinen et al., 1999; Dong et al., 2000; Girard et al., 2000; Kainu et al., 2000). In the majority of these studies, limited numbers of markers were used; thus, the development of a fine deletion map, which is key in identifying new tumor suppressor genes, could not be established.

Our group previously conducted several studies on allelic loss in esophageal squamous-cell carcinoma (ESCC) patients from Shanxi Province, China (Hu et al., 1999, 2000; Li et al., 2001). The results showed high frequencies of LOH on chromosome arm 13q, and several loci that were associated with a family history of upper gastrointestinal (UGI) cancer in ESCC (Hu et al., 1999, 2003; Li et al., 2001). In addition, two common regions of deletion were identified: one was located on segment 13q12.11 and another on 13q12.3-q13.1, where *BRCA2* is located. In the present study, we defined the deletion regions more fully, established

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a high-resolution deletion map on the entire long arm of chromosome 13, and compared LOH results according to family history of UGI cancer, cancer lifestyle risk factors, and clinical/pathologic characteristics of the tumors.

MATERIALS AND METHODS

Patients seen in 1995 and 1996 at the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, who were diagnosed with ESCC and considered candidates for curative surgical resection were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the U.S. National Cancer Institute (NCI). For this study, a total of 56 patients were selected, who had a histologic diagnosis of ESCC confirmed by pathologists at both the Shanxi Cancer Hospital and the NCI. None of the patients had prior therapy, and Shanxi was the ancestral home for all. Of the 56 ESCC patients studied, the 34 patients in Group 1 had a family history of UGI cancer (i.e., a first-, second-, or third-degree relative with cancer of the esophagus, gastric cardia, or body of stomach), and the 22 patients in Group 2 had no family history of any cancer. Information on demographic, clinical, and cancer lifestyle risk factors and a family history of cancer is shown in Table 1.

All 56 patients were previously examined for LOH on chromosome region 17p13.3-p11.1 (Huang et al., 2000) and underwent mutation testing for *TP53* (Hu et al., 2001), *BRCA2* (Hu et al., 2002), and *DICE1* (Li et al., 2003). Twenty-four of these patients were also examined for *ML-1* (now called *ATP8A2*) and *RNF6* gene mutations (Lo et al., 2002). All patients were followed up for ascertaining survival status as of 2003.

TABLE 1. Demographic, Clinical, and Lifestyle Characteristics of Study Subjects[a]

Characteristics	No. of cases	(%)
Gender		
Male	34	61
Female	22	39
Tumor location		
Upper	1	2
Middle	45	80
Lower	10	18
Tumor stage		
1	0	0
2	2	4
3	54	96
Tumor grade		
1	6	11
2	45	80
3	5	9
Metastasis		
Yes	21	38
No	33	59
Unknown	2	4
Smoker		
Yes	29	52
No	27	48
Drinks alcohol		
Yes	32	57
No	24	43
Eats pickled vegetables		
Yes	28	50
No	28	50
Eats scalding hot food		
Yes	28	50
No	28	50
Family history of UGI cancer[b]		
Yes	34	61
No	22	39

[a] $n = 56$; average age: 53.5 years.
[b] UGI cancer includes esophageal cancer, cardia cancer, and body of stomach cancer.

Biological Specimen Collection and Processing

Ten milliliters of venous blood was taken from each patient prior to surgery, and genomic DNA was extracted and purified with standard methods. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser Microdissection and Extraction of DNA

Tumor cells were microdissected under direct-light microscopic visualization by methods described previously (Emmert-Buck et al., 1996; Huang et al., 2000).

Markers, PCR, and LOH Reading and Interpretation

A total of 107 polymorphic microsatellite markers on chromosome bands 13q11-q34 were included in this study (http://www.cedar.genetics.soton.ac.uk/ldb/chrom13/gmap) (Human MapPair™, Research Genetics, Huntsville, Alabama), including 42 markers located on chromosome bands 13q11-q13, which were previously studied for allelic loss (Li et al., 2001), and 65 markers located on chromosome bands 13q12-q34 (Hu et al., 2003).

DNA extracted from tumor cells microdissected from the resection specimen and genomic DNA extracted from venous blood were studied for each patient. PCR reactions were carried out as previously described (Huang et al., 2000).

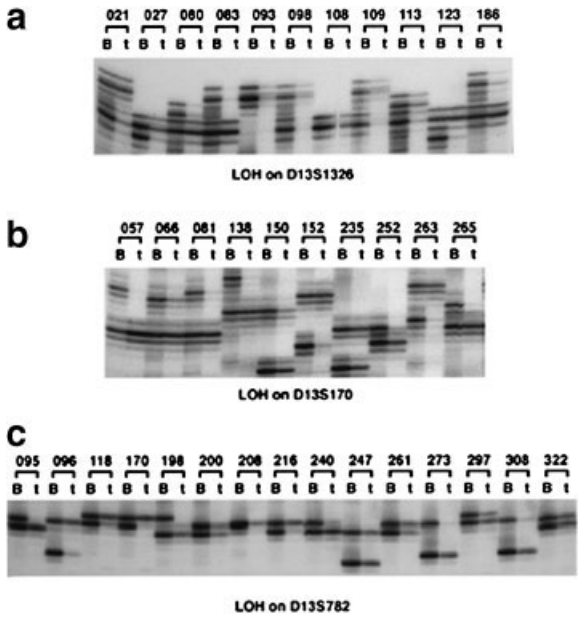

Figure 1. Allelic loss on markers D13S1326 (a), D13S170 (b), and D13S782 (c). Individual case numbers are shown at the top. B is normal DNA from blood, and t is DNA from tumor cells.

LOH was defined as either complete or nearly complete loss of a band in the tumor sample relative to the corresponding normal DNA (Fig. 1). All results were reviewed by two investigators independently (N.H. and M.E.-B.).

Calculation of the Frequency of Allelic Loss

Fractional allelic loss (FAL) is a measure of the extent of allelic loss in a given tumor sample (Vogelstein et al., 1989) and is calculated as the number of markers in a tumor showing any LOH divided by the total number of informative markers in the tumors. The frequency of FAL in each patient was classified as low (0–24%), medium (25–49%), high (50–74%), or very high ($\leq$75%).

The frequency of allelic loss was determined for each marker in which at least 11 of the 56 tumors (20%) were informative, and was calculated as the number of tumors with allelic loss at the marker divided by the number of informative tumors at the marker.

Nonrandom allelic loss was defined as LOH frequency $\geq$50% at a given locus, whereas random allelic loss represented an LOH frequency at a marker that was $<$50%.

A common region of deletion was determined by identifying the smallest regions of frequent LOH in a tumor, and was defined as a region where two or more contiguous markers showed $\geq$75% LOH, including intervening but uninformative markers between flanking regions of LOH.

Statistical Analysis

Differences in the pattern of tumor LOH frequencies between family-history-positive (Group 1) and -negative (Group 2) patients were first evaluated by use of Fisher's exact test and subsequently with a permutation test (Manly, 1997). The permutation test was based on the 10% trimmed mean of the χ^2 test statistic, comparing individual markers according to family history status. The null distribution of no difference in the pattern of LOH frequency across family history groups was obtained by randomly permuting the family history status 5000 times and evaluating the distribution of the (10% trimmed) mean χ^2 value. The permutation test provides a global test of the difference in the pattern of LOH frequency across family history status, thereby avoiding the inherent problem of multiple comparisons when differences at each marker location are tested. The association between the frequency of allelic loss and risk factors or clinical/pathologic characteristics was evaluated by *t* test (for continuous variables). For nominal variables, the χ^2, Mantel–Haenszel χ^2, or Fisher's exact test was used. Follow-up data were used for estimating 50% survival times and 5-year survival rates for allelic markers, and log-rank tests were used for evaluating differences in survival curves by allelic loss. All analyses were performed by use of Statistical Analysis Systems (SAS software, SAS Corp., Cary, North Carolina).

RESULTS

FAL in ESCC Patients

In this study, we used 107 polymorphic microsatellite markers covering the entire long arm of chromosome 13 to examine the 56 ESCC cases (Fig. 2). The heterozygosity rates for markers used in this study ranged from 0% to 98%. Allelic loss was detected in all 56 patients (100%) at one or more loci on chromosome arm 13q. The FAL ranged from 2% to 98% (Fig. 2). Of the 56 patients, 43% (24/56) had a very high frequency of FAL, 29% (16/56) had a high frequency of FAL, 7% (4/56) had a medium, and 21% (12/56) had a low frequency of FAL. Of the 34 patients with a family history of UGI cancer (Group 1), 28 (82%) had a very high or high frequency of FAL (Fig. 2a), compared with only 12 of the 22 (55%) patients without such a family history (Group 2) (Fig. 2b).

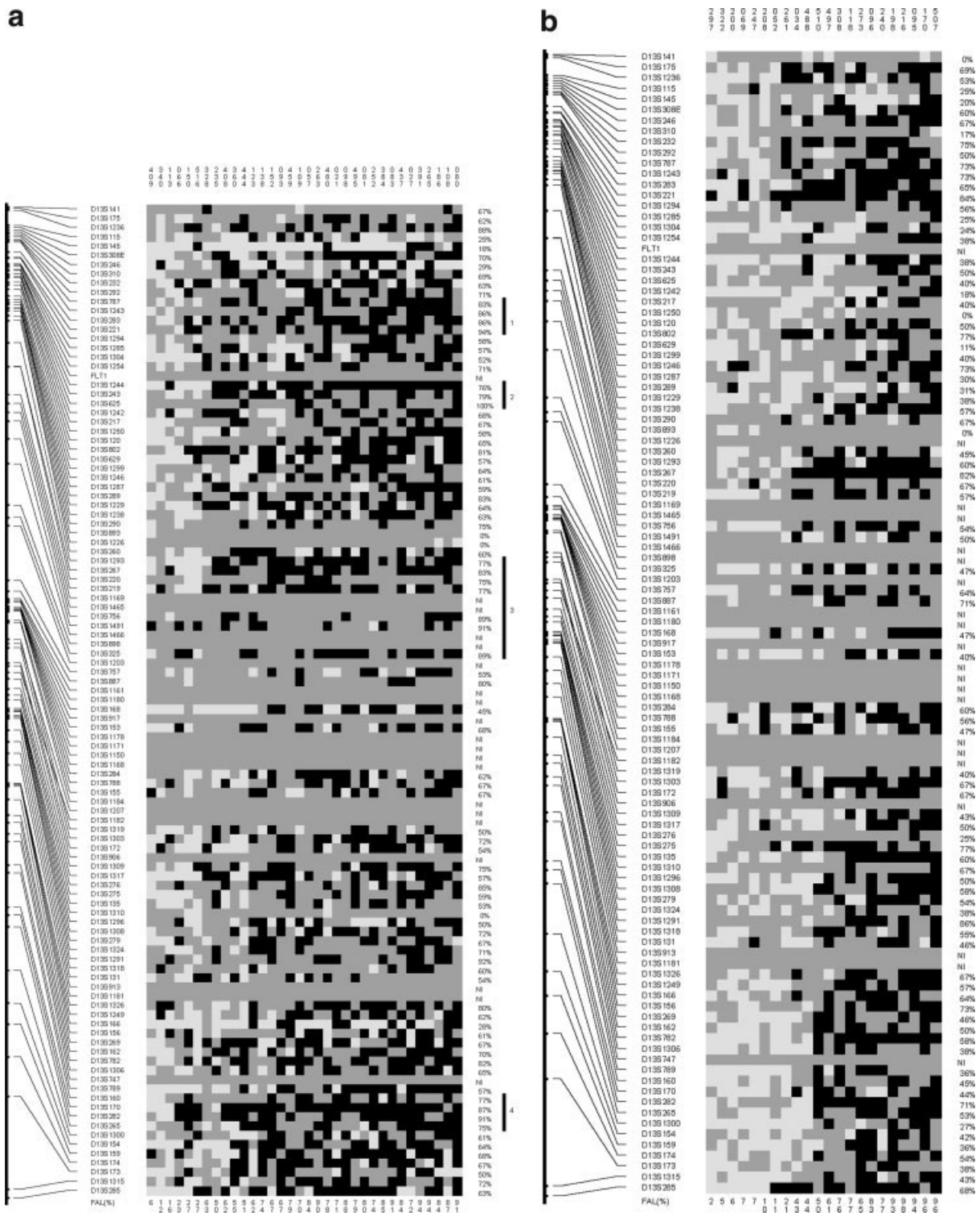

Figure 2. FAL map and deletion map for chromosome bands 13q11-q34 in esophageal squamous-cell carcinoma patients with (a) and without (b) a family history of UGI cancer. The numbers on the top are the patient's IDs, the numbers on the bottom are the FAL (%), the numbers on the left are the loci (markers), and the numbers on the right are the LOH frequencies. White represents cases with LOH $<50\%$, black represents cases with LOH $\geq 50\%$, and gray represents homozygous cases. The black vertical lines represent deletion regions 1–4 in (a).

TABLE 2. Summary of Deletion Regions in ESCC Patients with a Positive Family History of UGI Cancer

Deletion region	Location	Involved markers	Size of deletion region (Mb)	Genes of interest
I	13q12.11	D13S787, D13S1243, D13S283, D13S221	1.83	*LATS2, ZNF198, SCLC, GJB2, ML-1 (ATP8A2), RNF6*
II	13q12.12	D13S1244, D13S243, D13S625	0.671	*HRF*
III	13q12.3-q14.11	D13S1293, D13S267, D13S220, D13S219, D13S1169, D13S1465, D13S756, D13S1491, D13S1466, D13S898, D13S325	20.198	*AS3, BRCA2, DLEU1, DLEU2, RB1, GER, DMB, TPT1*
IV	13q31.1	D13S160, D13S170, D13S282, D13S265	10.374	

Association of Allelic Loss on Chromosome 13 with Family History

FAL differences between Groups 1 and 2

Both Fisher's exact and global permutation tests showed a significant difference between the mean FAL in Group 1 (67%) and that in Group 2 (50%) ($P = 0.035$ for Fisher's and $P = 0.03$ for permutation test), suggesting an association between LOH on chromosome arm 13q and family history in this high-risk area of China. Specifically, LOH at nine markers was significantly higher in Group 1 than in 2 ($P = 0.037$ for *D13S246*, $P = 0.01$ for *D13S1244*, $P = 0.045$ for *D13S625*, $P = 0.003$ for *D13S1242*, $P = 0.01$ for *D13S1250*, $P = 0.023$ for *D13S629*, $P = 0.008$ for *D13S289*, $P = 0.010$ for *D13S325*, and $P = 0.004$ for *D13S170*). Seven of these nine markers (7/9 = 78%) are located on chromosome band 13q12.

Deletion regions

Of the 107 markers, 20 were uninformative in all cases (Fig. 2), and four were informative in fewer than 20% of cases and so were dropped, leaving 83 markers for defining common regions of deletion. Four deletion regions (I, II, III, and IV in Table 2) were identified in Group 1, but none in Group 2 (Table 2, Fig. 2). The deletion regions were 1.83 Mb (13q12.11), 0.671 Mb (13q12.12), 20.198 Mb (13q12.3-q14.11), and 10.374 Mb (13q31.1) in size (Table 2).

Single markers with very high frequency of LOH

Several single markers showed very-high-frequency LOH, but were not located in a deletion region. Ten such high-frequency LOH markers were identified in Group 1 cases, whereas six of these markers were present in Group 2 cases (Table 3, Fig. 2). Two of these markers showed very high frequencies in both Groups 1 and 2 (81% and 77% for *D13S802* on 13q12.12, and 92% and 86% for *D13S1291* on 13q21.3, respectively).

TABLE 3. Summary of Single Markers with Very-High-Frequency LOH (≥75%) in ESCC Patients

No	Locus	Location	LOH (%)[a]
Group 1			
1	*D13S1236*	13q11	88 (14/16/34)
2	*D13S802*[b]	13q12.12	81 (21/26/33)
3	*D13S290*	13q12.12	75 (6/8/29)
4	*D13S289*	13q12.13	82 (14/17/33)
5	*D13S887*	13q14.2	80 (4/5/30)
6	*D13S1309*	13q21.1	75 (15/0/30)
7	*D13S276*	13q21.2	85 (11/13/31)
8	*D13S1291*[b]	13q21.31	92 (11/12/27)
9	*D13S1326*	13q21.33	80 (16/20/34)
10	*D13S782*	13q22.1	82 (18/22/32)
Group 2			
1	*D13S232*	13q12.11	75 (9/21/21)
2	*D13S221*	13q12.11	84 (16/19/22)
3	*D13S802*[b]	13q12.12	77 (10/13/22)
4	*D13S267*	13q12.3	82 (14/17/22)
5	*D13S275*	13q21.2	77 (10/13/22)
6	*D13S1291*[b]	13q21.31	86 (6/7/14)

[a]Values in parentheses indicate the number of cases with loss, the number of informative cases, and the number of total cases, respectively.
[b]Present in both Group 1 and Group 2.

Nonrandom and random LOH

Nonrandom LOH was found in 94% (78/83) and 55% (47/83) of informative markers in Groups 1 and 2, respectively (Fig. 2), a difference that was statistically significant ($P < 0.001$, Fisher's exact test).

Association of Allelic Loss on Chromosome 13 with Clinical Characteristics and Lifestyle Factors

LOH at four markers was significantly associated with lymph node metastasis (*D13S1309* on 13q12.11, $P = 0.014$; *D13S310* on 13q12.1, $P = 0.046$; *D13S1254* on 13q12.12, $P = 0.022$; and *D13S1249* on 13q21.33, $P = 0.030$; Fisher's exact test). Three of these markers are located on 13q12, suggesting that markers on chromosome band 13q12 may be useful as biomarkers of survival in follow-up studies. LOH at five markers, three of which are located on 13q12, was associated with a

higher tumor grade (*D13S175* on 13q11, $P = 0.039$; *D13S246* on 13q12.1, $P = 0.028$; *D13S1250* on 13q12.12, $P = 0.042$; *D13S1254* on 13q12.12, $P = 0.046$; and *D13S172* on 13q21.1, $P = 0.010$; χ^2 test). Further, LOH at three markers, including two on 13q12, was associated with tumor location (*D13S1242* on 13q12.12, $P = 0.006$; *D13S1287* on 13q12.13, $P = 0.005$; and *D13S1308* on 13q21.21, $P = 0.001$; Mantel–Haenszel test).

There were no significant associations between LOH on chromosome 13 and lifestyle factors, including eating pickled vegetables and drinking alcohol, except that LOH on D13S1309 was associated with smoking ($P = 0.03$, Fisher's exact test).

Association of Allelic Loss at *D13S246* and *D13S1250* with Family History and Tumor Grade

Because LOH at D13S246 and D13S1250 was significantly associated with both family history and tumor grade, we examined the joint relation of these variables. Although family history and tumor grade were unrelated ($\chi^2_{2df} = 1.03$, $P = 0.597$), the relation of tumor grade to LOH at both of these markers was modified by family history status. Within grade 2 (the only category of tumor grade with sufficient cases to be stratified by family history status), only 21% (5/24) of family-history-positive cases had LOH at *D13S*, compared with 63% (5/8) in family-history-negative cases ($\chi^2_{5df} = 11.52$, $P = 0.42$). The reverse was true for *D13S1250* among grade 2 cases: 57% (8/14) of family-history-positive cases had LOH, compared with 0% (0/6) in family-history-negative cases ($\chi^2_{4df} = 11.95$, $P = 0.018$).

Association of Allelic Loss on Chromosome 13 with Survival

To determine whether LOH in general or LOH at specific markers predicted especially aggressive or benign behavior, we also examined the relationship between survival time and LOH. Although no significant associations were observed between markers and survival time or rate, several observations were made that merit further investigation. Overall, the average 50% survival time for persons with LOH at any marker was 29.4 months (versus 45.1 months for cases without LOH), and the 5-year survival rate was 28% versus 33% for these two groups ($P > 0.05$ for both comparisons). For individual markers, the greatest differences in survival time and rate for persons with (versus without) LOH of a marker were an apparent benefit for 13S1293 LOH (50% survival time, 48.0 months with LOH versus 30.4 months without LOH; 5-year survival, 42% versus 27%, respectively; log-rank test $P = 0.157$) and reduced survival for 13S1491 LOH (50% survival time, 10.1 months with LOH versus 45.3 months without LOH; 5-year survival, 20% versus 36%, respectively; log-rank test $P = 0.140$).

DISCUSSION

The present study describes a detailed analysis of high-resolution allelotyping data from 56 ESCC patients by use of 107 microsatellite markers that covered the entire long arm of chromosome 13. The results showed frequent allelic loss at most markers on this chromosome in these patients from China. Analysis also showed higher FAL in cases with a family history of UGI cancer, an observation that was even stronger when only nonrandom allelic-loss rates were compared. These differences in regard to family history status do not appear to be related to or explained by other factors such as age, gender, clinical characteristics (including tumor location, tumor grade, tumor stage, or lymph node metastasis), or lifestyle risk factors (including tobacco use, alcohol use, consumption of pickled vegetables, or very hot food) (Huang et al., 2000), and they suggest that greater allelic loss, particularly nonrandom loss, on chromosome 13 in patients with a positive family history of UGI cancer may be associated with a genetic predisposition. To assess further the significance of the difference in tumor LOH frequency between family-history-positive and family-history-negative cases on chromosome arm 13q, we previously performed a similar global permutation test on chromosome arm 17p, using results from the 30 microsatellite markers (Huang et al., 2003), and found no difference in terms of family history status ($P = 0.22$, global permutation test). Taken together, these results suggest that the higher frequencies of FAL and nonrandom allelic loss on chromosome arm 13q are related to a genetic susceptibility in ESCC patients with a family history of UGI cancer among this high-risk Chinese population.

In this study, we modified our criteria for defining a common region of deletion. The new criteria, which are stricter than those we used before (LOH $\geq$70%) (Li et al., 2001), were defined as two or more contiguous markers that showed $\geq$75% LOH, including intervening non informative markers between flanking regions of LOH. With these more stringent criteria, we identified four deletion regions in ESCC patients with a positive family history, but none in patients without a family history. It is well known that chromosomal regions with frequent allelic loss may point to major susceptibility

genes, and that allelic loss in somatic cells of patients with a genetic predisposition may pinpoint those loci that harbor recessive germ-line mutations (Kainu et al., 2000; Hu et al., 2003).

To test the hypothesis that ESCC patients with a positive family history carry a unique allelic variant on chromosome 13, we evaluated the DNA sequence of several candidate genes on this chromosome. We analyzed two genes, *ML-1* (now called *ATP8A2*) and *RNF6*, that are located within 800 kb of each other on 13q12.11 (deletion region I in Table 2) by direct sequencing in 34 of the 56 ESCC patients. Three somatic mutations in the *RNF6* gene were detected in ESCC patients without a family history of UGI cancer (3/24, 13%), but no mutation in *ML-1* (*ATP8A2*) was detected in any of the patients (Lo et al., 2002). This suggests that *RNF6* is a potential tumor suppressor gene involved in the pathogenesis of ESCC.

We have also examined two other candidate tumor suppressor genes in or near this deletion region. First, the entire coding region of *BRCA2*, located in deletion region III defined (Table 2), was examined in 126 ESCC patients. The 56 patients studied here were evaluated by use of PCR-SSCP and DNA sequencing (Hu et al., 2002), whereas the other 70 patients were studied by direct full sequencing (Hu et al., 2004). We found that the cumulative frequency of germ-line *BRCA2* mutations in ESCC patients in these two studies was 12%; interestingly, all nine of these mutations were found in patients with a positive family history (Hu et al., 2004). Second, we analyzed mutations in the *DICE1* gene in this same group of 56 patients. Although *DICE1* is not located within deletion region III (Table 2), it is nearby, on 13q14.12-q14.2 (GenBank No. AF097645). Three somatic mutations were identified in three patients, including two in family-history-positive cases (Li et al., 2003). Taken together, these findings suggest that mutations in the candidate tumor suppressor genes *BRCA2* and *DICE1* do occur in ESCC, and that germ-line and somatic variants of these two genes on chromosome bands 13q12 and q14 may be related to ESCC in cases with a positive family history.

Harada et al. (1999) studied LOH on 13q in ESCC by using 18 markers and found that LOH at *D13S171* was significantly associated with lymph node metastasis in ESCC. In our study, we found that LOH at four markers (but not *D13S171*) was significantly associated with lymph node metastasis in ESCC, including three markers located at 13q12.11-q12.12. Based on the OMIM (Online Mendelian Inheritance in Man) gene map, this region contains several interesting candidate genes, including: *LATS2* (Yabuta et al., 2000; Ishizaki et al., 2002), *ED2* (ectodermal dysplasia 2) (Radhakrishna et al., 1997), *GJB2* (gap junction protein beta-2, also known as *CX26* or connexin 26 gap junction protein) (Mignon et al., 1996), *GJA3* (gap junction protein alpha-3, also known as *CX46* or connexin 46 gap junction protein) (Mignon et al., 1996), and *ZNF198* (zinc finger protein 198) (Xiao et al., 1998). However, determining the possible roles of these genes in lymph node metastases of ESCC will require further analysis. We also observed that LOH for a number of markers was associated with tumor grade and smoking; however, because the number of informative markers for these evaluations was limited, further data are needed for determination of the potential importance of these associations.

In summary, allelic deletion patterns on chromosome 13 were analyzed in depth in 56 ESCC patients by use of 107 microsatellite markers spanning the entire length of the chromosome. Tumor LOH frequency was significantly higher in cases with a positive family history than in those who were family-history-negative. In addition, we identified four common deletion regions in these family-history-positive patients, but none in patients without such a family history. These results suggest that very high frequency LOH on chromosome 13 is related to ESCC in cases with a positive family history, and that these patients may carry a unique allelic variant on this chromosome.

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